A redistribution of actin and myosin IIA accompanies Ca^{2+}-dependent insulin secretion

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Abstract The study addressed the functional link between remodelling of the actomyosin cytoskeleton in pancreatic β-cells and the regulation of insulin secretion. Confocal microscopy revealed that myosin heavy chain (MHC) IIA co-localized very well with filamentous (F)-actin in RINm5F cells but MHCIIB did not. Subcellular localization of MHCIIB was not altered by stimulation with 30 mM KCl (which evokes Ca^{2+}-dependent insulin secretion). In contrast MHCIIA redistributed in a manner similar to F-actin, especially towards the apical surface, but also away from peripheral regions towards cell contact points on the basal surface. Finally, Ca^{2+}-dependent insulin secretion was inhibited by stabilization of actin filaments with jasplakinolide. The results support a role for the MHCIIA/actin cytoskeleton in regulating insulin secretion.

Key words: Exocytosis; Cytoskeleton; Pancreatic β-cell; Myosin heavy chain; Jasplakinolide

1. Introduction

A rise in the cytosolic free Ca^{2+} concentration is a key event in the stimulation of insulin secretion [1–3]. The subsequent activation of Ca^{2+}-dependent protein kinases is thought to be crucial to the secretory process but the substrates of these protein kinases are very poorly characterized [4–6]. However we have recently identified myosin II heavy chain (MHC) as one such substrate in pancreatic islets and insulin-secreting RINm5F cells [7,8] which was phosphorylated chiefly on threonine residues at times preceding the peak of secretion [7]. Whereas both MHCIIA and MHCIIB were present in β-cells, threonine phosphorylation occurred mainly on MHCIIA. Threonine phosphorylation of MHC was subsequently shown to correlate with dissolution of the cortical actin web of neuronal cells [9]. This is consistent with a postulated requirement for disruption of a cytoskeletal meshwork in order to allow final access of secretory vesicles to the plasma membrane [9–13]. However evidence in support of this hypothesis for insulin secretion is limited to observations that agents which disrupt actin also enhance secretion [14–17]. The function of myosin in this context is poorly understood in β-cells, because of a prior emphasis on myosin's other role: as a motor protein driving transport of secretory granules [16,18]. Motor function is chiefly controlled by phosphorylation of regulatory light chains (RLCs) which are also present with MHC in the myosin II complex [19,20]. However we have recently shown that phosphorylation of RLCs in β-cells is constitutive and (in contrast to MHC phosphorylation) not further augmented by insulin secretagogues [8].

The aim of the current study was to reassess the involvement of the actin cytoskeleton in the secretory response in pancreatic β-cells. Our results indicate that cytoskeletal remodelling is required for insulin secretion, and are consistent with a model in which phosphorylation of MHCIIA facilitates granular access to the plasma membrane.

2. Materials and methods

RINm5F cells were maintained in RPMI 1640 as adherent monolayers [7]. Studies of insulin secretion were exactly as described [8] except that cells were cultured overnight in 24-well plates at 10^6 cells/well. Experimental incubations were in modified Krebs–Ringer bicarbonate buffer (KRB) containing 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 2.8 mM glucose, 10 mM HEPES (pH 7.4) and 0.5% (w/v) bovine serum albumin (BSA). Jasplakinolide (Molecular Probes) was dissolved in dimethyl sulfoxide and this solvent was also added to control incubations (0.1% final). Insulin secreted into the medium during a 15 min incubation was measured by radioimmunoassay. For confocal microscopy, cells were cultured overnight at $0.1–0.15\times10^6$cells/0.4 ml/well in 8-well chamber slides (Nalge-Nunc International). Experimental additions were made in 0.4 ml KRB at 37°C for 5 min. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS)/0.1% (v/v) Triton X-100 at room temperature for 30 min, and then washed twice in PBS/0.05% (v/v) Tween 20 and blocked for ~30 min in PBS/1% BSA. Myosin was detected using primary antisera diluted 1:20, with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Amersham). These primary antisera were raised against the C-termini of rat non-muscle MHCIIA and MHCIIB, respectively [21], and like those made to corresponding regions of *Xenopus* and human non-muscle MHC isoforms, and which have been extensively characterized [22,23], their specificity has been confirmed previously by immunoblotting [7,21]. Cells were then mounted in 90% (v/v) glycerol, 10% (v/v) PBS containing 1 mg/ml *p*-phenylenediamine. No immunofluorescence was observed in fixed cells incubated with the FITC-conjugated secondary antibody alone. Filamentous (F)-actin was detected using Texas Red-linked phalloidin diluted 1:200 (Sigma) and, in co-localization studies, this was added after the myosin and the FITC-conjugated secondary antibodies. Cells were visualized at 60× magnification using a Sarastro 2000 CLSM laser scanning confocal microscope from Molecular Dynamics. Optical sections were taken at 0.39 μM intervals.

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Abbreviations: MHC, myosin heavy chain; RLC, regulatory light chain; KRB, Krebs–Ringer bicarbonate buffer; FITC, fluorescein isothiocyanate; F-actin, filamentous actin; PBS, phosphate-buffered saline

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3. Results

The subcellular distribution of MHCIIA, MHCIIB and F-actin in RINm5F cells was investigated using confocal microscopy. Composite images (Fig. 1A,B) revealed that although both MHC isozymes were fairly widely distributed, MHCIIA was concentrated in cell peripheries, whereas these areas were largely devoid of MHCIIB. These differences are even more

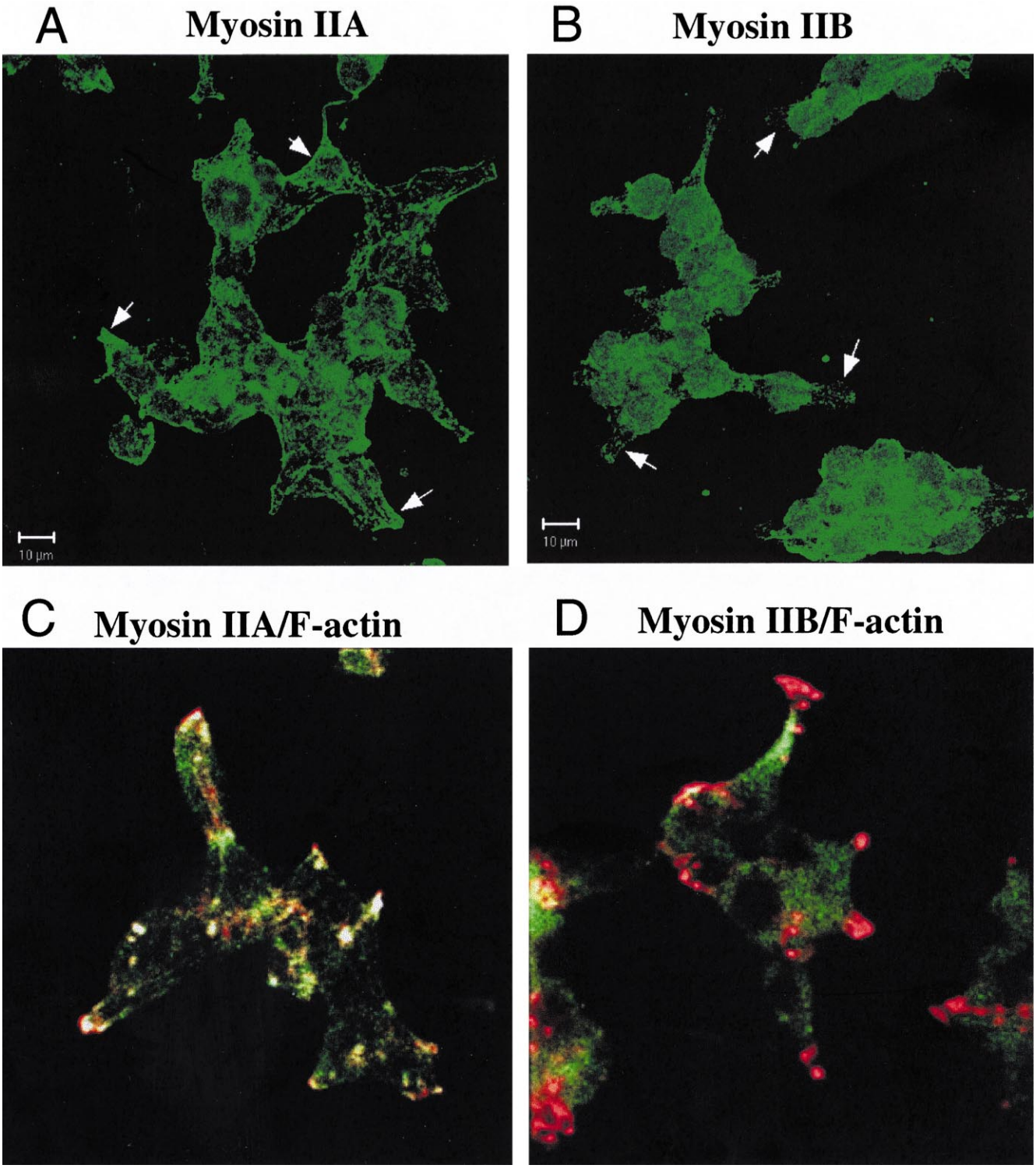

Fig. 1. Subcellular localization of myosin IIA/IIB and co-localization with F-actin. RINm5F cells were fixed and exposed to specific myosin IIA (A and C) or myosin IIB antibodies (B and D) and an FITC-conjugated secondary antibody. Confocal microscopy was undertaken at 60× magnification. (A and B) Horizontal (*z*-) sections were made through the cells at a step size of ∼0.39 μm and the images are presented as a projection series. The arrows highlight differential distribution of myosin IIA and IIB in leading edges. (C and D) Cells were also labelled with Texas Red-conjugated phalloidin to visualize F-actin. The images presented are single (∼0.39 μm) sections of the basal region of the cells.

apparent on the basal cell surface (Fig. 1C,D) where F-actin co-localizes with MHCIIA, but not MHCIIB, at cell peripheries and regions of cell to cell contact. Indeed there appeared to be very little co-localization of F-actin and MHCIIB at all in these cells.

Fig. 2A shows a more complete analysis of the experiments from which the composite image shown in Fig. 1C was taken. Distributions of MHCIIA and F-actin are shown individually (as well as merged) on both basal and apical surfaces of unstimulated cells. On the basal surface (panel d) F-actin was concentrated at peripheries and areas of cell contact, but was more diffuse on the apical cell surfaces (panel a). MHCIIA staining (panels b and e) was generally similar to that of actin, as confirmed by the merged images (panels c and f) most obviously on the basal surface, but also in sub-membrane regions on the apical surface. Upon stimulation with KCl (Fig. 2B) there was a marked concentration of myosin (cf. panels b and h) and especially actin (panels a and g) around the apical surface. Although less extensively investigated, the nutrient stimulus glyceraldehyde (10 mM) induced similar alterations (not shown). Yellow areas of merged fluorescence confirm a degree of co-localization of MHCIIA and actin in these areas, but some regions of red signal also suggest that co-localization is not uniform (panel i). Cell stimulation also

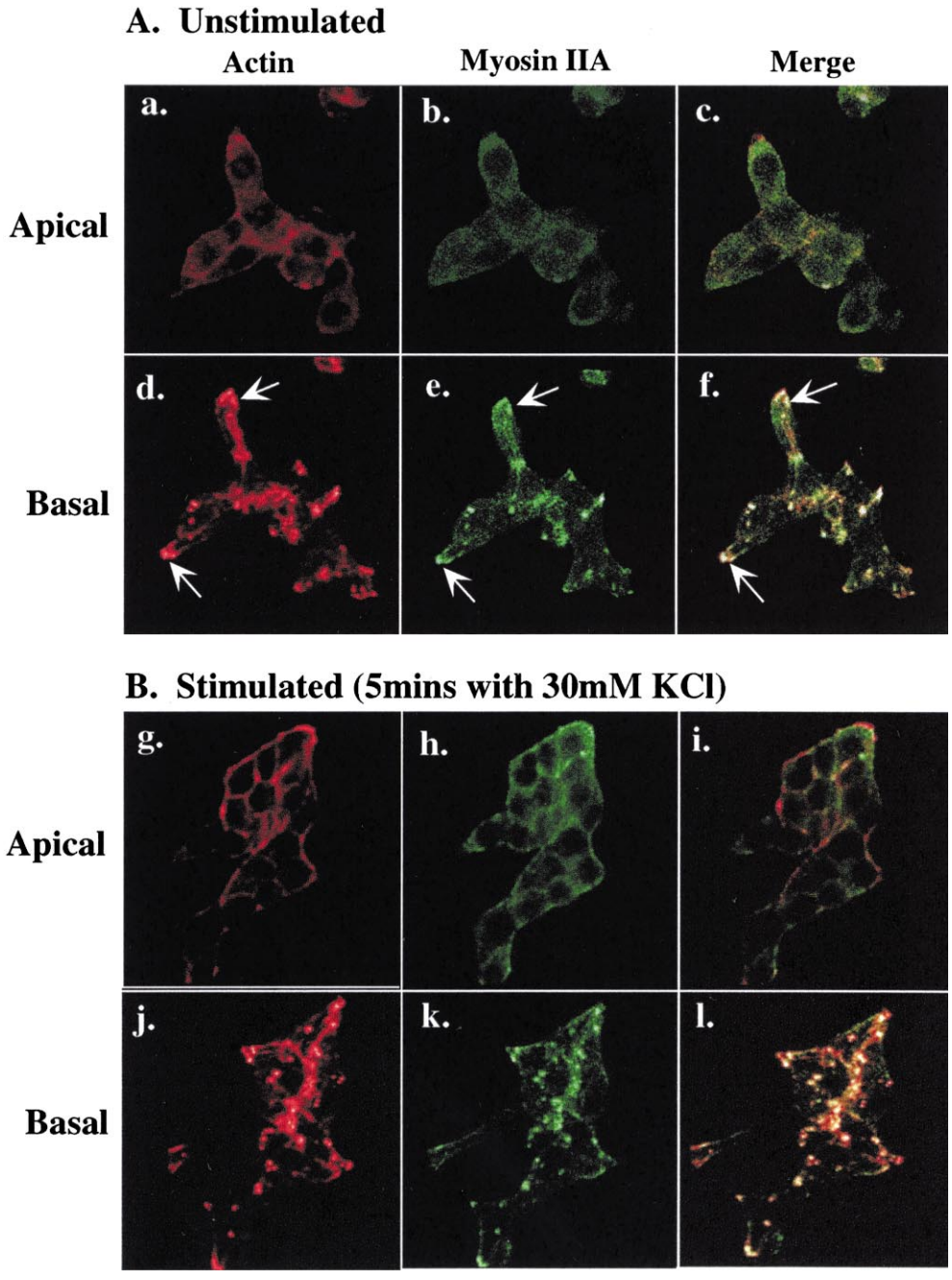

Fig. 2. Myosin IIA and F-actin co-localization. Unstimulated (A) or stimulated (B) were dual labelled to visualize F-actin (red staining, left panels) and myosin IIA isoform (green staining, middle panels). Overlap of the fluorophores appears yellow in the merged images (right panels). The images presented (60× magnification) are single (~0.39 μm) sections of the apical and basal region of the cells. The arrows highlight the presence of F-actin and myosin IIA in leading edges on the basal surface.

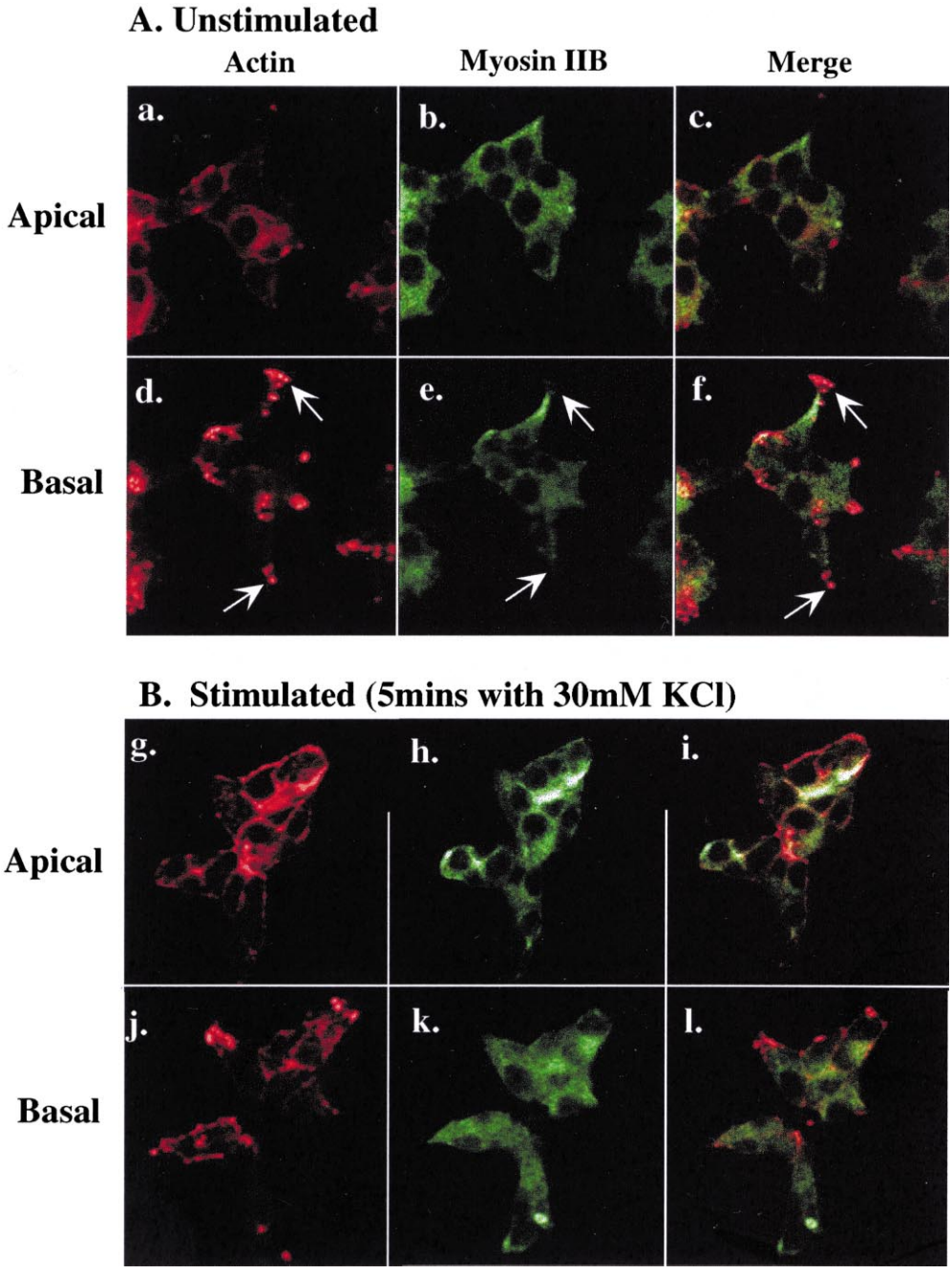

Fig. 3. Lack of Myosin IIB and F-actin co-localization. Images (60× magnification) were generated as described in the legend to Fig. 2 except that middle panels represent staining for myosin IIB. The arrows highlight the presence of F-actin but absence of myosin IIB in leading edges on the basal surface.

caused a subtle consolidation of both actin and myosin in the areas of cell to cell contacts, and away from the peripheries, on the basal surface. This is most evident in the merged images (cf. panels f and l). Note also that the strong co-localization of MHCIIA and F-actin on this surface is retained upon cellular stimulation (panel l).

Corresponding analysis of MHCIIB distribution (Fig. 3A) revealed poor co-localization with F-actin on both apical and basal surfaces. Stimulation with KCl (Fig. 3B) caused a redistribution of F-actin at the apical surface (panel g) and a concentration in areas of cell to cell contacts on the basal surface (panel j) but this was not accompanied by any discernible rearrangement of MHCIIB (panels b versus h). Likewise there was virtually no co-localization of the two proteins at either surface under stimulated conditions (panels i and l).

The next experiments examined the effect on secretion of jasplakinolide, which stabilizes actin filaments [24]. While not affecting basal insulin secretion, this compound inhibited by more than 50% the response to stimulatory KCl concentrations (Fig. 4). As previously documented [24], actin was only poorly visualized using Texas Red-conjugated phalloidin in jasplakinolide-treated cells (not shown) since these two agents compete with each other for binding to F-actin [24]. This confirms that the effects of jasplakinolide on secretion are

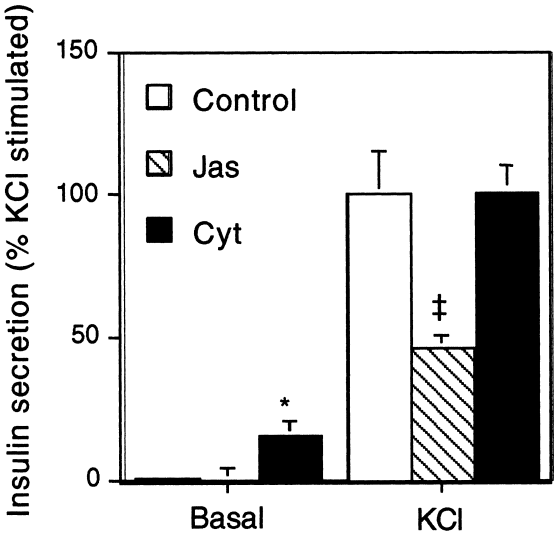

Fig. 4. Effect of modulation of F-actin polymerization on insulin secretion from RINm5F cells. Insulin release from basal or KCl-stimulated RINm5F cells was measured over 15 min in the presence or absence of jasplakinolide (Jas) or cytochalasin B (Cyt). Results are means ± S.E.M. from three independent experiments each assayed in quadruplicate and are expressed as percentages of the incremental KCl response. Statistical significance was calculated using Student's *t*-test where * represents $P < 0.05$ versus basal control, and ‡ indicates $P < 0.05$ versus KCl control.

likely to be due to actin binding in RINm5F cells. Conversely cytochalasin B, which disrupts actin filaments (14–17), enhanced basal secretion to a level of approximately 15% of KCl response (Fig. 4) or more than double basal (not shown).

4. Discussion

This study provides a number of novel observations to support the hypothesis that F-actin remodelling helps regulate the release of insulin granules from pancreatic β-cells. Crucially, we demonstrate for the first time that F-actin is actually redistributed in these cells in response to a secretory stimulus. This was not detected in a previous study using fluorescence microscopy on a hamster insulinoma cell line [17]. By exploiting the more sensitive technique of confocal microscopy, and by focusing independently on both apical and basal surfaces rather than full transmission images, we have now clearly demonstrated alterations in F-actin staining upon stimulation. The second novel observation is that jasplakinolide which prevents F-actin remodelling inhibits stimulated, but not basal, secretion. This clearly implies that dynamic alterations in the actin cytoskeleton are actually necessary for a full secretory response, and extends the interpretation of earlier studies using cytochalasin which suggested that such alterations were sufficient to promote secretion [14–17]. This extended conclusion is also consistent with the recent findings that exocytosis in patch-clamped, mouse β-cells was decreased by inhibition of scinderin, an F-actin severing protein [25].

Our results are therefore broadly consistent with the hypothesis, elucidated using a number of secretory cells [9–13], that final access of secretory granules to the plasma membrane requires dissolution of a defined cortical actin web [9–13]. However the cortical actin web of RINm5F cells, as visualized in the current study, is not as well-defined as in some other secretory cell types, nor is its dissolution as pronounced on stimulation. This does not preclude involvement of the cytoskeletal changes described here in controlling insulin secretion, since granular access could equally (and more efficiently) be regulated by a more subtle remodelling of cytoskeletal complexes, rather than their complete fragmentation. Examples of this might include formation of gaps in the actin meshwork and/or a retraction of the cell web away from exocytotic sites. However this would be difficult to visualize unless more specific protein markers for such cytoskeletal 'hot-spots' became available. In this context it should also be stressed that phalloidin does not stain all F-actin pools [26] and that perhaps a functional cortical actin web might be better detected in the future using antisera to specific actin isoforms, or other actin binding proteins [26,27].

The demonstration that MHCIIA and MHCIIB are differentially localized in pancreatic β-cells is another novel finding. Furthermore the observations that MHCIIB was diffusely expressed, co-localized poorly with phalloidin-stained actin, and was not apparently redistributed upon cellular stimulation, render it an unlikely candidate for controlling secretion. On the other hand MHCIIA exhibited a more discrete localization, especially on the basal cell surface where it was highly co-localized with F-actin on cell peripheries. Upon stimulation, MHCIIA accompanied the redistribution of F-actin that occurred on both the basal and apical cell surfaces. These findings are of potential interest given that Ca^{2+}-dependent stimulation of pancreatic β-cells results in a more pronounced threonine phosphorylation of MHCIIA than MHCIIB [7,28]. Although we have no direct evidence of a causal link between phosphorylation and subcellular redistribution of MHCIIA-associated F-actin, such a link would at least be consistent with the strong correlation between MHC threonine phosphorylation and cytoskeletal remodelling found in neuronal N1E-115 cells [10]. A role for MHC phosphorylation in controlling the cross-linking of actomyosin filaments is also directly supported by experiments with invertebrate models [19,29].

In conclusion we have demonstrated that Ca^{2+}-dependent insulin secretion is accompanied by morphological changes in F-actin distribution in RINm5F cells, and that depolymerization of actin filaments is necessary for secretion to proceed. In addition there are stimulus-dependent changes in the localization of MHCIIA but not MHCIIB. The results are consistent with the actin/myosin IIA cytoskeleton playing an important role in the regulation of insulin secretion, potentially in controlling the final access of secretory granules for docking with the plasma membrane.

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